ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 1990, p. 1088–1093
0066-4804/90/061088-06$02.00/0

Vol. 34, No. 6

Mechanism of Action of Lomefloxacin

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Received 5 September 1989/Accepted 28 February 1990

The inhibition of supercoiling activity of reconstituted *Escherichia coli* DNA gyrase by lomefloxacin, ciprofloxacin, and norfloxacin was determined. The concentrations of quinolones needed to inhibit DNA synthesis in *Escherichia coli*, *Enterobacter cloacae*, *Serratia marcescens*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* were also measured. The kinetics of uptake of [^{14}C]lomefloxacin and unlabeled lomefloxacin into whole cells of *E. coli* KL-16 and *S. aureus* NCTC 8532 and the induction of RecA in *E. coli* GC2241 were assayed. All strains had wild-type susceptibilities to quinolones. The concentration of quinolones needed to inhibit DNA synthesis by 50% correlated with the MIC for members of the family *Enterobacteriaceae* and *P. aeruginosa*. The concentration of quinolones needed to inhibit DNA synthesis by 50% for late-logarithmic-phase *S. aureus* also correlated with the MIC, unlike the data from early-logarithmic-phase cultures. *E. coli* and *S. aureus* showed a similar pattern of uptake kinetics of [^{14}C]lomefloxacin and unlabeled lomefloxacin, indicating that the difference in the susceptibilities of the two species is probably due to different target site affinities. Essentially, lomefloxacin was less active than ciprofloxacin and ofloxacin and had activity similar to those of norfloxacin and enoxacin.

Lomefloxacin (SC-47111; NY-198) is a new difluorinated quinolone. Its in vitro activity has been compared with those of other quinolones and has been shown to have activity similar to that of ofloxacin (4, 11), with the MIC for 90% of the members of the family *Enterobacteriaceae*, *Pseudomonas aeruginosa*, and staphylococci tested being between 0.25 and 4 μg/ml. Clinical isolates and laboratory mutants that were resistant to nalidixic acid and that had decreased susceptibilities to fluoroquinolones were also less susceptible to lomefloxacin. No cross resistance between strains that were resistant to other classes of antimicrobial agents and lomefloxacin was observed (12). The pH has been shown to affect the MIC of lomefloxacin markedly (as with other quinolones) but not the MBC. Following an oral dose of 200 mg, a maximum concentration in serum of 2 μg/ml was achieved (S. Kamidoro, A. Fujii, H. Nagata, and J. Ishigani, 15th Int. Congr. Chemother., abstr. no. 1340, 1987), and in another study, a single oral dose of 400 mg gave a peak concentration in serum 1 h after administration of 4.7 μg/ml, with a mean elimination half-life of 7 h (11). A break-point concentration of 4 μg/ml has been suggested by the manufacturers (G. D. Searle and Co. Ltd.). We compared the activity of lomefloxacin with those of established quinolones in several strains of *Escherichia coli*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Serratia marcescens*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*.

MATERIALS AND METHODS

Bacterial strains. *E. coli* NCTC 10538, KL-16, AB1157, and GC2241; *P. aeruginosa* NCTC 10662 and PAO1, *S. aureus* NCTC 8532; *E. cloacae* NCTC 10005; *K. pneumoniae* NCTC 9633 and *S. marcescens* NCTC 10211 were used throughout the study. All strains except *E. coli* AB1157 and GC2241 were obtained from the National Collection at the Public Health Laboratory, London, United Kingdom. *E. coli* AB1157 and GC2241 were obtained from S. Casaregol, Department of Biochemistry, University of Leicester, Leicester, United Kingdom.

Antibiotics. The following antimicrobial agents were studied and were obtained from the indicated sources: lomefloxacin, Searle Laboratories, High Wycombe, United Kingdom; nalidixic acid, Sterling Research Group Europe, Guildford, United Kingdom; enoxacin, Parke-Davis Warner-Lambert, Eastleigh, United Kingdom; norfloxacin, Merck, Sharpe and Dohme Research Laboratories, Hoddesdon, United Kingdom; fleroxacin, Hoffmann-La Roche, Basel, Switzerland; ofloxacin, Hoechst Laboratories, Uxbridge, United Kingdom; and ciprofloxacin, Bayer AG, Wuppertal, Federal Republic of Germany. All compounds were made up and used according to the instructions of the manufactures.

Susceptibility determinations. The susceptibilities of the strains to the compounds were studied by a routine agar plate dilution method. For all strains, the inocula were prepared by growing the strains overnight in Iso-Sensitest broth (Oxoid Ltd., Basingstoke, United Kingdom) to yield a viable count of about 10^9 CFU/ml. The Iso-Sensitest agar plates containing doubling dilutions of the antibiotics were inoculated by transferring 1 μl of an undiluted culture or a 1:100 dilution of the overnight culture to the surface of the agar with a multipoint inoculating device (Denley-Tech, Billingshurst, United Kingdom). The final inocula on the plates were therefore 10^4 and 10^6 CFU, respectively. All plates were incubated aerobically at 37°C overnight. The MIC of the antibiotic was defined as that concentration (in micrograms per milliliter of agar) at which no more than two colonies were detected. For the higher inoculum a slight haze of growth was ignored.

Killing kinetics. The bactericidal activity of each agent was examined by a viable count method. The test antibiotic was added at logarithmic dilutions to flasks containing an exponential-phase culture, and at timed intervals portions were removed. Each sample was centrifuged at 10,000 × *g* at 37°C

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for 10 min; and the cell pellet was suspended in phosphate-buffered saline (pH 7, 37°C) and then diluted as necessary, spread onto agar, and incubated aerobically at 37°C. This washing step removed excess quinolone from samples containing antibiotic concentrations that would be sufficiently high to inhibit growth on agar; this step did not affect the determination (data not shown). All viable count determinations were performed in triplicate.

Induction of RecA protein. The method of RecA protein induction was essentially that described previously (2). Briefly, parallel flasks containing 100 ml of *E. coli* GC2241 (*E. coli* AB1157 containing a gene fusion between *recA* and *gal*) at mid-exponential phases were inoculated with the test antibiotic at logarithmic dilutions (0, 0.001 to 1,000 μg/ml) and incubated aerobically with shaking at 37°C. At 15-min intervals (up to 2 h), portions were withdrawn and assayed for β-galactosidase (5). Enzyme concentrations (in units per milliliter) were calculated from the formula given by Casaregola et al. (2). An arbitrary value of 60 U/ml was chosen to estimate the minimum inducing concentration of quinolone (8).

Measurement of DNA gyrase-mediated supercoiling. The assay of DNA gyrase-mediated supercoiling activity was kindly performed by A. Maxwell (Department of Biochemistry, University of Leicester) essentially as described by Mizuuchi et al. (7). Briefly, subunits A and B of DNA gyrase from wild-type *E. coli* N4186 containing pmK50 or pmK47 were purified to apparent homogeneity. The subunits were combined to form a reconstituted DNA gyrase, and the supercoiling activity of this preparation was determined essentially as described by Reece and Maxwell (9) by using plasmid pBR322 DNA as a substrate. The inhibition of supercoiling by quinolones was determined by adding doubling dilutions of quinolones to the reconstituted DNA gyrase and plasmid mixture and incubating this combination of ingredients for 60 min at 25°C. The reaction was terminated by the addition of equal volumes (30 μl) of chloroform and isoamyl alcohol on ice. Sample buffer (10 μl) was added and mixed well. A 20-μl mixture containing about 250 ng of DNA was electrophoresed on an agarose gel. After electrophoresis the gel was stained with ethidium bromide and scanned. The concentration of quinolone needed to inhibit supercoiling by 50% (IC_{50}) was then determined.

Assay of DNA synthesis. For members of the family *Enterobacteriaceae* and *S. aureus*, the assay of DNA synthesis was essentially that described previously (10). Briefly, exponentially growing, 10-ml cultures of the *Enterobacteriaceae* (in Davis minimal broth [Difco Laboratories, Detroit, Mich.] supplemented with 0.4% Casamino Acids [Difco], 0.2% glucose, and 0.01% aneurine hydrochloride) or *S. aureus* in Iso-Sensitest broth were grown to an optical density at 675 nm (OD_{675}) of 0.2 to 0.3. To each tube quinolone (0, 0.001 to 1,000 μg/ml), 1 μCi of [^{3}H]thymidine, and 100 μl of deoxyadenosine (5 mg/ml; Sigma Chemical Co., St. Louis, Mo.) was added and incubated aerobically with shaking at 37°C. After 30, 60, or 90 min, duplicate 1-ml portions were withdrawn into ice-cold tubes containing 100 μl of 50% trichloroacetic acid with thymidine (1 mg/ml), and the tubes were incubated on ice for 10 min. The contents of each tube were then filtered onto Whatman GFC filters (Whatman, Inc., Clifton, N.J.) under vacuum and washed with 1% trichloroacetic acid. The filters were dried and scintillation counted. Exponential-phase *P. aeruginosa* cells (grown in supplemented minimal medium as described above) were grown to an OD_{470} of 0.5 and assayed as described previously (1). Briefly, to duplicate tubes containing 50 μl of fresh prewarmed medium, 1 μCi of [^{3}H]adenine, and quinolone, 200 μl of culture was added and incubated aerobically with shaking at 37°C for 4 min. The reaction was terminated by adding 100 μl of 3 N NaOH and 110 μl of 1% EDTA, and the tubes were incubated overnight at 37°C. Bovine serum albumin (final concentration, 40 μg per tube) was added, and the DNA was precipitated with 50% ice-cold trichloroacetic acid. The contents of each tube were collected onto cellulose nitrate filters, washed, dried, and scintillation counted.

Measurement of uptake. Two techniques were used to measure the uptake kinetics of lomefloxacin. (i) Exponential-phase cultures of *E. coli* KL-16 and *S. aureus* in Iso-Sensitest broth were grown to an OD_{675} of 0.2 to 0.3, and the uptake of [^{14}C]lomefloxacin was measured essentially as described previously (3a). Briefly, a 10 ml of culture was centrifuged and suspended in 10 ml of sterile prewarmed fresh broth containing a magnetic flea and allowed to equilibrate at 37°C in a water bath on a heated magnetic stirrer. Fifty microliters of culture was removed at 5 min before the addition of lomefloxacin to determine the viable count. The reaction was initiated by the addition of [^{14}C]lomefloxacin (9.18 μCi/mg; G. D. Searle and Co. Ltd.) to duplicate tubes at final concentration of 16 μg/ml. At timed intervals, duplicate portions were removed and filtered through nylon membrane filters (Ultipore; Pall Trinity Micro Corp., Cortland, N.Y.). The filters were washed and dried, and the activity was determined by scintillation counting. In parallel, the nonspecific binding of the quinolone to the filters was measured by adding [^{14}C]lomefloxacin to Iso-Sensitest broth and by filtering and washing as described above. This was performed in triplicate. The radioactive counts of the filters of [^{14}C]lomefoxacin alone were averaged and subtracted from the values obtained for the filtered cells in the uptake assay. (ii) Uptake was also measured by a fluorometric assay (3). The assay was modified and was performed essentially as follows. Parallel flasks containing 500 ml of Iso-Sensitest broth were inoculated with *E. coli* KL-16 or *S. aureus*, and the organisms were allowed to grow to an OD_{660} of 0.7 to 0.8. Each flask of bacterial cells was harvested at 4°C and was suspended in 20 ml of 50 mM sodium phosphate buffer (pH 7), washed once, and suspended to 20 ml. The bacterial suspension was transferred to a 100-ml sterile flask and allowed to equilibrate at 37°C for 10 min. Lomefloxacin was added to each flask to final concentrations of 1, 5, 10, and 16 μg/ml. At timed intervals, 500-μl samples were withdrawn and added to 1 ml of sodium phosphate buffer on ice. Viable counts were performed in parallel. The samples were centrifuged immediately in a microcentrifuge (Microcentaur; MSE) at 13,000 × *g* and washed once with sterile buffer. To each pellet 1 ml of 0.1 M glycine hydrochloride (pH 3) was added, mixed well to obtain a smooth suspension, and incubated at 25°C for 2 h. The suspensions were centrifuged for 10 min at 13,000 × *g*, and the supernatant was removed to fresh tubes and recentrifuged for 5 min. The fluorescence of the supernatant was measured at an excitation of 291 nm and an emission of 456 nm and was compared with a standard curve of 0.01 to 10 μg of lomefloxacin per ml in 0.1 M glycine hydrochloride (pH 3).

RESULTS

Susceptibility and bactericidal activity. Table 1 shows the activities of quinolones against all the strains used in this study. Each organism showed the typical susceptibility of a normal wild-type (susceptible) strain for all agents. *E. coli*

TABLE 1. Susceptibilities of *Enterobacteriaceae*, *P. aeruginosa*, and *S. aureus* to quinolones

Strain[a]	MIC (μg/ml)						
	Lomefloxacin	Nalidixic acid	Enoxacin	Norfloxacin	Fleroxacin	Ofloxacin	Ciprofloxacin
E. coli NCTC 10538	0.25	4	0.25	0.12	0.25	0.12	0.015
E. coli KL-16	0.12	4	0.5	0.06	0.12	0.12	0.015
E. coli AB1157	0.12	4	0.25	0.12	0.12	0.03	0.03
E. coli GC2241	0.12	8	0.12	0.12	0.25	0.12	0.015
P. aeruginosa NCTC 10662	4.00	>512	2.00	2.00	2	4.00	0.5
P. aeruginosa PAO1	4.00	>512	8.00	4.00	2	4.00	1.00
S. aureus NCTC 8532	2.00	>512	4.00	2.00	8.00	2.00	0.5
E. cloacae NCTC 10005	0.06	2	1.00	0.25	0.5	0.12	0.008
K. pneumoniae NCTC 9633	0.25	8	0.25	0.25	0.25	0.25	0.03
S. marcescens NCTC 10211	0.25	4	0.25	0.25	0.25	0.15	0.06

[a] Strains were used at an inoculum of 10^6 CFU.

GC2241, a derivative of *E. coli* AB1157, had a susceptibility similar to that of its progenitor. Against all strains, lomefloxacin was more active than nalidixic acid and had activity similar to those of enoxacin, norfloxacin, fleroxacin, and ofloxacin; but it was less active than ciprofloxacin.

A similar pattern of activity was observed in the killing kinetic studies. When the viable count data obtained after 60 min of exposure to quinolone were plotted against the concentration (Fig. 1), a typical dose-response curve of fluoroquinolones was observed, with an optimum bactericidal concentration of lomefloxacin against *E. coli* AB1157 of 10 μg/ml.

Induction of RecA protein. Figure 2 shows the induction of RecA by lomefloxacin compared with that by enoxacin. With an increase in concentration, each agent caused more induction up to a maximum concentration, above which there was less induction. For both agents there was no significant induction below 0.01 μg/ml. Maximum induction was obtained at 0.1 μg of lomefloxacin per ml and 1 μg of enoxacin per ml, with each value comparing well with the MICs (0.12 and 0.5 μg/ml, respectively). No induction of RecA above 10 μg of either agent per ml was observed. The values obtained for the maximum concentrations of all agents needed to induce RecA were similar to the MICs and mirrored the order of activity (data not shown).

Inhibition of DNA gyrase-mediated supercoiling. The ability of reconstituted DNA gyrase from *E. coli* to supercoil plasmid pBR322 DNA was examined by agarose gel electrophoresis. Lomefloxacin and norfloxacin at 1.5 μg/ml and ciprofloxacin at 0.5 μg/ml inhibited 50% of the in vitro supercoiling. There was no distinction between lomefloxacin and norfloxacin, even though these agents had different MICs (0.12 and 0.06 μg/ml, respectively). The values obtained were all well above the MICs of all three agents.

Inhibition of DNA synthesis. Figure 3 shows the inhibition of DNA synthesis in intact cells of *E. cloacae*, *S. marcescens*, and *K. pneumoniae* by lomefloxacin after 90 min of exposure. DNA synthesis in all three strains was inhibited 90% (IC_{90}) by 1 μg of lomefloxacin per ml (Table 2), which is well below the break-point concentration. The IC_{50} data for all strains and agents are also shown in Table 2 and correlated well with the MICs.

Figure 4 shows the inhibition of DNA synthesis in *P. aeruginosa* NCTC 10662 and PAO1. As *P. aeruginosa* does not incorporate thymidine, an alternative method of assessing DNA synthesis with adenine was used (1). Both strains had identical susceptibilities to lomefloxacin and showed very similar levels of inhibition of DNA synthesis. As with the members of the family *Enterobacteriaceae*, the IC_{50} correlated with the MIC.

Figure 5 shows typical data obtained for the inhibition of DNA synthesis in *E. coli* and *S. aureus* after 90 min of exposure to quinolones for cultures at the early and late logarithmic phases. For *E. coli*, the IC_{50} and the IC_{90} of

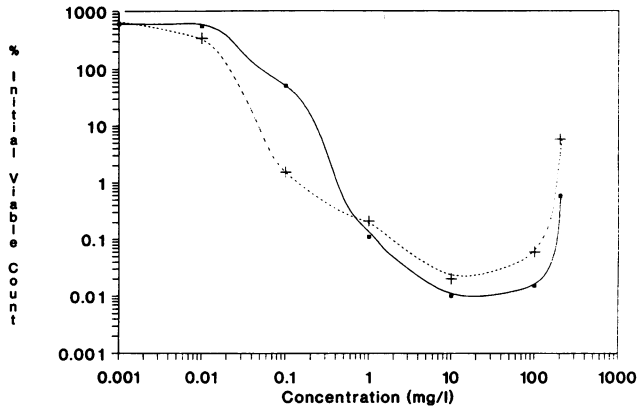

FIG. 1. Survival of *E. coli* AB1157 after 60 min of exposure to quinolones. Symbols: ■, lomefloxacin; +, norfloxacin.

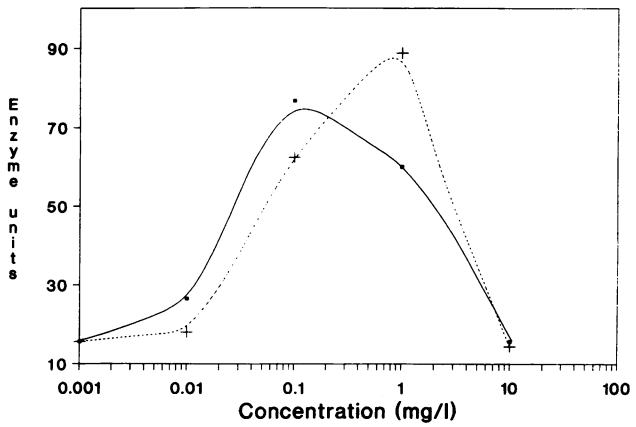

FIG. 2. Induction of RecA in *E. coli* GC2241 after 80 min of exposure to quinolones. Symbols: ■, lomefloxacin; +, enoxacin.

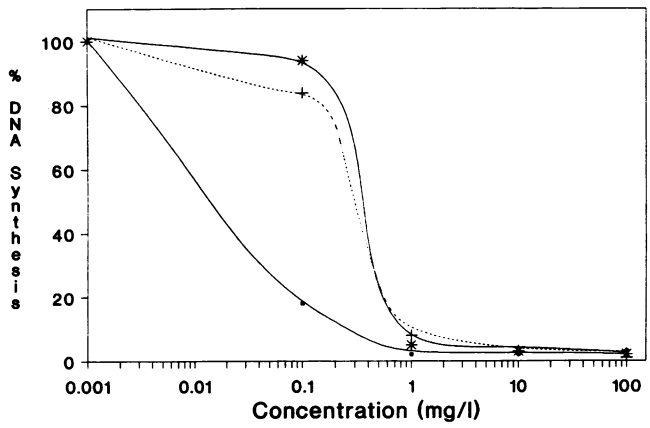

FIG. 3. Inhibition of DNA synthesis in members of the family *Enterobacteriaceae* by lomefloxacin after 90 min of exposure. Symbols: ■, *E. cloacae*; +, *S. marcescens*; *, *K. pneumoniae*.

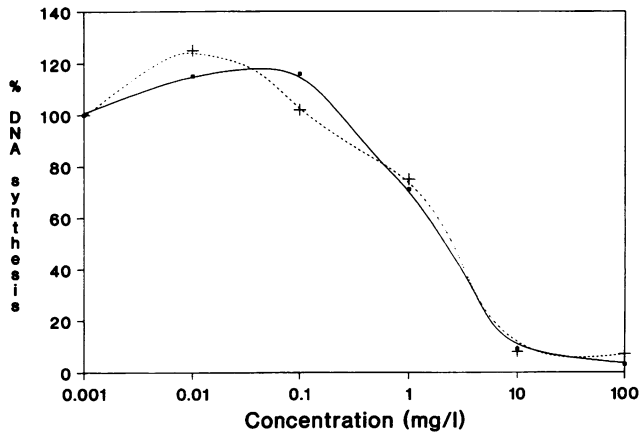

FIG. 4. Inhibition of DNA synthesis in *P. aeruginosa* by lomefloxacin after 4 min of exposure. Symbols: ■, NCTC 10662; +, PAO1.

lomefloxacin were of the same order of magnitude, regardless of the growth stage of the culture, with IC_{90}s of 0.12 and 0.37 μg/ml obtained for the early and late logarithmic phases, respectively. However, the values obtained for *S. aureus* NCTC 8532 differed. The data obtained from an early-logarithmic-phase culture showed that DNA synthesis was very susceptible to quinolone action, and neither the IC_{50} nor the IC_{90} bore any relation to the MIC. However, when the assay was performed with a late-logarithmic-phase culture, the IC_{50}s that were obtained correlated well with the MIC.

Uptake of lomefloxacin. The uptake of [^{14}C]lomefloxacin into logarithmic-phase cultures of *E. coli* KL-16 and *S. aureus* is shown in Fig. 6. To compare uptakes by the two species, the scintillation count data (disintegrations per minute) were normalized by using the viable count of each culture taken at 5 min before the addition of radiolabeled lomefloxacin. After 30 min of exposure to 16 μg of lomefloxacin per ml, there was a 1-log-unit drop in the viable count. Similar data were obtained from replicate experiments that showed that *E. coli* KL-16 and *S. aureus* NCTC 8532 have similar kinetics of uptake. This was also confirmed by a fluorometric assay of lomefloxacin uptake (Fig. 6B and C). In this study in which we compared two different species, it was necessary to normalize the data obtained by a common denominator, such as dry weight of the bacterial cells or viable count. We found that at similar OD_{675} or OD_{660} values there were a different number of viable *S. aureus* cells (10^9) compared with the number of viable *E. coli* cells (10^8). The viable count was directly proportional to the dry weight (data not shown). The fluorometric assay was more versatile and indicated that rapid uptake occurred for both species, and maximum uptake was achieved by 2 min. *S. aureus* achieved a higher steady-state concentration than *E. coli* KL-16 did; however, this phenomenon appears to be strain- and quinolone-dependent (unpublished data). Saturation of the uptake system for lomefloxacin was not observed for either species (data not shown). A consistent and reproducible finding was that, by using a radiolabeled quinolone uptake assay or a fluorometric assay, similar patterns of uptake were observed. Mutants of *E. coli* lacking OmpF, OmpC, and PhoE$^-$ showed minimal uptake (data not shown).

DISCUSSION

The susceptibility data for all the strains used in this study compared well with previously published data (4, 12). The concentrations of norfloxacin and ciprofloxacin needed to inhibit the supercoiling activity of reconstituted *E. coli* DNA

TABLE 2. IC_{50}s and IC_{90}s of quinolones

Strain	IC (μg/ml)									
	Lomefloxacin		Nalidixic acid		Norfloxacin		Ofloxacin		Ciprofloxacin	
	50%	90%	50%	90%	50%	90%	50%	90%	50%	90%
E. coli NCTC 10538	0.1	1.0	10.0	72.0	0.058	0.5	0.076	0.32	0.016	0.074
E. coli KL-16	0.075	0.37	2.8	22.0						
E. coli AB1157	0.15	0.58	3.6	30.0						
P. aeruginosa NCTC 10662	1.5	8.6			1.1	5.2	3.3	17.0	0.34	3.0
P. aeruginosa PAO1	2.5	7.0			0.56	5.2	3.6	60.0	0.63	15.0
S. aureus NCTC 8532										
Early log phase	0.045	0.4								
Late log phase	1.5	8.0	2,800	4,800						
E. cloacae NCTC 10005	0.037	0.17	0.26	3.5						
K. pneumoniae NCTC 9633	0.33	0.62	2.2	16.0						
S. marcescens NCTC 10211	0.28	0.8	1.8	5.6						

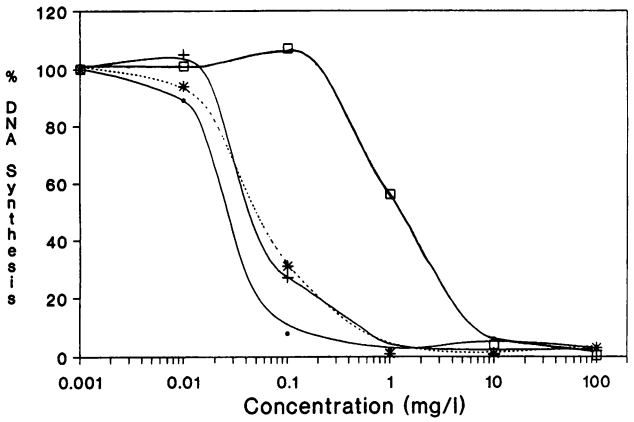

FIG. 5. Inhibition of DNA synthesis in *E. coli* and *S. aureus* after 90 min of exposure to lomefloxacin. Symbols: ■, *E. coli*, early log phase; *, *E. coli*, late log phase: +, *S. aureus*, early log phase; □, *S. aureus*, late log phase.

gyrase compared well with previously published data (6). In this study, lomefloxacin was found to have inhibitory activity similar to that of norfloxacin, although the IC_{50}s were above the MICs. Inhibition of bacterial DNA synthesis by quinolones is thought to occur via inhibition of DNA gyrase, and measurement of the inhibition of DNA synthesis reflects the inhibition of DNA gyrase. Therefore, values obtained from the assay of whole cells without perturbing any essential enzymes (such as may happen during purification of DNA gyrase subunits) may, in fact, give more valid data. In this study, DNA synthesis IC_{50}s correlated well with the MIC data, and for *E. coli* AB1157 these values also compared well with the maximum RecA-inducing concentration. Measurements of DNA synthesis by using intact cells assume that there is no significant permeability barrier to the quinolone or the nucleotide. In this study, it was assumed that because the strains that were used showed the typical susceptibilities of susceptible wild-type strains, no significant permeability barrier to quinolones existed. This was confirmed by the excellent correlation between the MIC and the IC_{50}. For strains lacking OmpF it is difficult to correlate the IC_{50}s and MICs for DNA synthesis unless the cells are permeabilized with toluene before the assay for DNA synthesis (unpublished data).

The uptakes of [^{14}C]lomefloxacin into *E. coli* KL-16 and *S. aureus* NCTC 8532 showed similar patterns, indicating that quinolones may enter gram-positive bacteria by a mechanism similar to that by which they enter gram-negative bacteria. The DNA synthesis IC_{50}s and IC_{90}s for *S. aureus* with a late-logarithmic-phase culture compared with those with an early-logarithmic-phase culture were different, unlike the values obtained for *E. coli*. This phenomenon is undergoing further investigation. The IC_{50} for *S. aureus* that correlated with the MIC was much higher than the IC_{50} and MIC of lomefloxacin for *E. coli*, and as similar uptakes were observed, the difference in susceptibilities is most likely due to the different affinities of lomefloxacin for the target site, DNA gyrase, in the two organisms.

In this study we showed that lomefloxacin, like other quinolones, inhibits bacterial DNA synthesis, presumably by inhibition of DNA gyrase. Damage to DNA by lomefloxacin is also shown by the induction of RecA, which is an essential enzyme in the SOS response (the DNA repair mechanism). It is a potent bactericidal agent and shows activity similar to those of norfloxacin and ofloxacin.

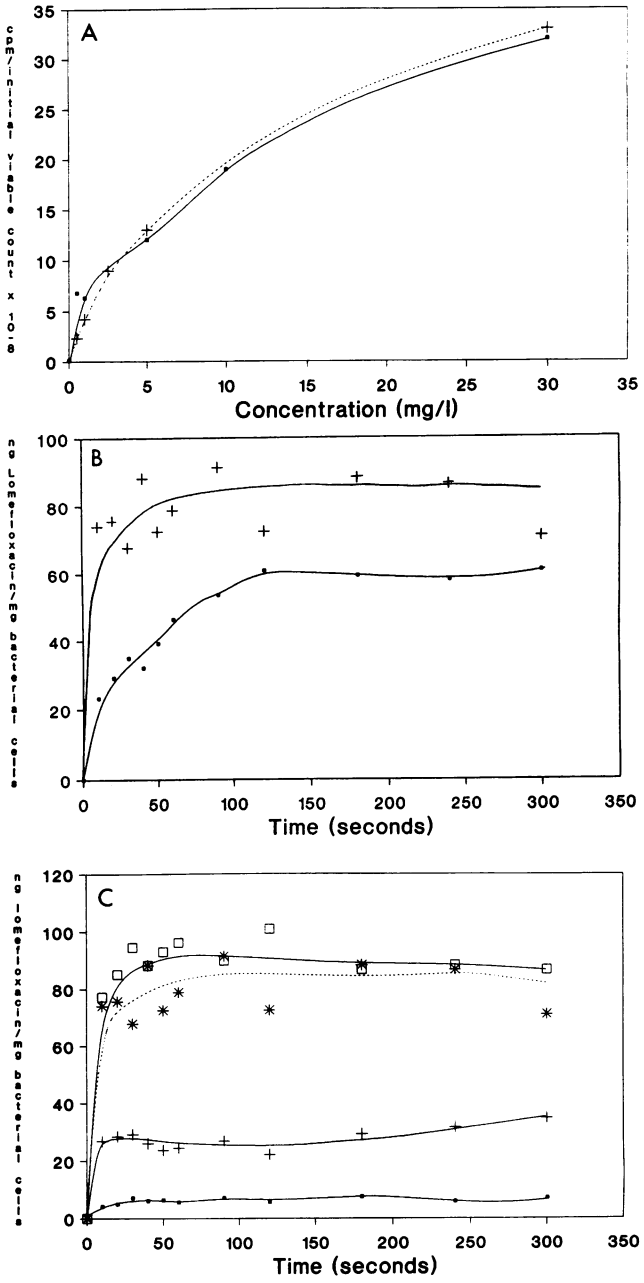

FIG. 6. (A) Uptake of 16 μg of [^{14}C]lomefloxacin per ml at 37°C. (B) Uptake of lomefloxacin (10 μg/ml) by fluorometric method at 37°C. Symbols for panels A and B: ■, *E. coli*; +, *S. aureus*. (C) Uptake of lomefloxacin by *S. aureus* at 1 μg/ml (■), 5 μg/ml (+), 10 μg/ml (*), and 16 μg/ml (□) at 37°C.

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